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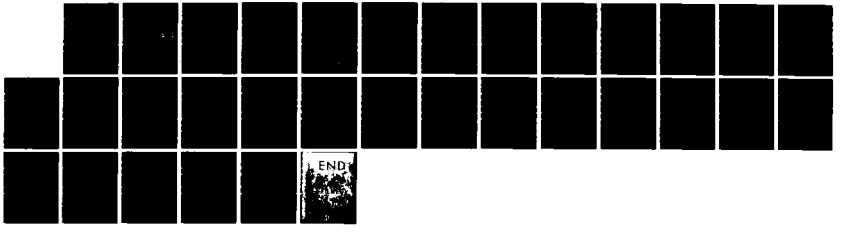

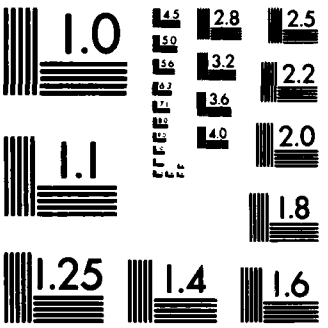

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PATHOGENESIS OF DENGUE VACCINE VIRUSES IN MOSQUITOES

Third Annual Report

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The dengue-2 vaccine virus (S-1) and its parent virus (PR-159) were compared for their ability to infect orally, to replicate in, and subsequently to be transmitted by Aedes aegypti mosquitoes. The vaccine virus was markedly less efficient in its ability to infect mosquitoes orally. After ingesting infectious bloodmeals containing 3.7 to 8.2 $\log_{10}$ $TCID_{50}$/ml of the respective viruses, 56% (220/396) of the mosquitoes

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became orally infected with the parent virus contrasted to 16% (66/397) for the vaccine virus. None of the 16 infected mosquitoes transmitted the vaccine virus, while 14% (3/22) of the mosquitoes transmitted the parent virus. The vaccine virus remained temperature sensitive (39°C) after orally infecting and replicating in Ae. aegypti mosquitoes.

An improved in vitro assay for transmission of dengue parent and vaccine viruses is being developed. Using an oil-charged capillary feeding system, saliva can rapidly and reliably be collected from even moribund mosquitoes. This technique will greatly facilitate studies on the assessment of vector competence.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Summary

Studies were continued to compare the efficiency of oral infection, mode of development, and transmission potential of dengue-2 parent and candidate vaccine viruses in Aedes aegypti and Aedes albopictus mosquitoes. Both strains were capable of oral infection of the vector species; however, the Aedes albopictus mosquitoes seemed to be more susceptible to oral infection. After ingesting 8.2 to 4.2 $\log_{10}$ $TCID_{50}$ per ml of the parent (PR 159) virus, 66% (46/68) of the Aedes aegypti became infected; in contrast, 97% (68/70) of the Aedes albopictus became infected. After ingesting the same amounts of the vaccine (S-1) virus, 20% (18/88) of the Aedes aegypti became infected; however, 65% (40/65) of the Aedes albopictus became infected. The vaccine strain was less infective for both vector species. In expanded studies using approximately the same infective doses, 56% (220/396) of the Aedes aegypti mosquitoes became infected with the parent virus, but only 16% (66/397) of the mosquitoes became infected with the vaccine virus.

The oral infectious $dose_{50}$ for the parent virus was between 5.4 and 5.7 $\log_{10}$ MID_{50}. The OID_{50} for the vaccine virus was 7.2 $\log_{10}$ MID_{50}. Thus, it required more than 100 times more vaccine than parent virus to infect 50% of the mosquitoes.

In oral transmission trials, 14% (3/22) of mosquitoes infected with the parent virus transmitted. In contrast, none (0/16) of the mosquitoes infected with the vaccine virus transmitted.

Pathogenesis studies were conducted to determine the anatomic basis of the reduced transmission capability of the vaccine-infected mosquitoes. Viral antigen was frequently detected in mosquito midgut tissues but not in secondary target organs. Thus the vaccine virus seemed less efficient in dissemination from midgut tissues than the parent virus.

The vaccine virus remained stable during mosquito passage. Although plaque size was somewhat altered, no large plaques were detected after mosquito passage, nor did the virus change in temperature sensitivity.

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I. Statement of the problem

The purpose of this research project is to determine if dengue parental and candidate vaccine viruses differ in their respective abilities to infect, to replicate in, and to be transmitted by Ae. aegypti and Ae. albopictus mosquitoes. Attenuated candidate vaccines and parental strains of dengue-1, dengue-2, and dengue-4 viruses will be compared in their vector-virus interactions.

The second, and related, objective of this research project is to determine if attenuated vaccine strains revert to virulence after mosquito passage. Should a live dengue vaccine be capable of infecting and subsequently be transmitted by mosquitoes to a new vertebrate and should the vaccine revert to virulence as a consequence of mosquito passage, then a natural infection cycle could be initiated.

The rationale for this project is that the temperature sensitive (ts) vaccine strains of the dengue viruses which are attenuated for man will also be modified in one or more parameters of vector-virus interactions. The hypotheses are 1) the vaccine strains will be less capable than parental strains in vector infection, 2) vaccine strains will differ from parent strains in their mode of development, 3) the vaccine strains will be less efficiently transmitted than parent strains, and 4) that the small plaque ts mutant virus populations will remain stable upon passage in vector mosquitoes.

II. Background

Dengue is of great tactical significance to the military because large numbers of troops can become incapacitated in a short period of time. Attenuated dengue vaccines have been developed at WRAIR.

The dengue-2 S-1 vaccine and PR-159 parent and the dengue-1 parent and TP 56 vaccine strains are the subject of this project report. The S-1 vaccine was derived from the serum of patient PR-159 of Puerto Rico (Eckels et al., 1976). The virus was passaged 6 times in Lederle certified African green monkey kidney cells. Passage 6 is designated the parent strain and S-1 represents the progeny of a small plaque derived from the parent strain (Eckels et al., 1980). The S-1 clone is ts, titers 340 times higher in LLC-MK_2 cells than in mice, does not produce viremia in rhesus monkeys, produces barely detectable viremia in chimps and in man (Bancroft, et al., 1981; Harrison, et al., 1977; Scott, et al., 1980). Only 2 of 114 Ae. aegypti mosquitoes fed on viremic volunteers became infected, but did not transmit the virus after 21 days incubation (Bancroft et el., 1982).

The dengue-1 candidate vaccine, TP 56, passage 28, was derived from a human serum isolate obtained during an epidemic on the island of Nauru in the South Pacific. It was passaged in fetal rhesus lung cells. The TP 56 candidate vaccine is ts, small plaque, and produces a low level viremia in rhesus monkeys. It has not been tested in man.

Ideally a vaccine should not produce viremia, but if it does, it is reasonable to expect that the vaccine strain will infect mosquitoes poorly and will be inefficiently transmitted. This was demonstrated with the 17D yellow

fever vaccine (Davis et al., 1932; Roubaud and Stefanopoulo, 1933; Peltier et fever vaccine (Roubaud et al., 1937; Whitman, 1939), French neurotropic yellow al., 1939), mouse-adapted dengue type 1 (Sabin, 1948), African green monkey kidney-adapted dengue type 2 (Price, 1973), and an attenuated Japanese encephalitis vaccine virus (Chen and Beaty, 1982). Sabin (1948) showed that attenuated dengue, passed through mosquitoes, did not revert to pathogenicity for man, and Chen and Beaty (1982) demonstrated that the attenuated Japanese encephalitis vaccine did not revert to mouse virulence after mosquito passage.

Thus, even if the vaccine did develop sufficient viremia to infect vectors, there would be little likelihood that the virus would be transmitted and that it would revert to virulence.

III. Approach

The working hypothesis was made that the ts candidate vaccine viruses and the parental wild-type viruses would behave differently in vector mosquitoes. To test this hypothesis the efficiency of oral infection of each parental and vaccine candidate strain was to be determined in dose response studies. Sequential 30-fold dilutions of the virus preparations were to be used to infect groups of a minimum of 10 sibling mosquitoes per dilution. Such studies would also provide information about the optimal infective dose for the transmission and pathogenesis studies; doses much greater than the threshold could obscure differences in infectivity between the vaccine and parental viruses.

Mosquitoes for studies to determine infection rates, extrinsic incubation periods, and rates of oral transmission were to be infected via engorgement on known titer blood-virus mixtures. Vector competence studies and especially dose-response studies are greatly facilitated by the use of artificial bloodmeals. In previous contract periods an efficient technique was developed to orally infect Aedes spp. mosquitoes with artificial meal systems (see Materials and Methods).

Vector-virus interactions were to be further investigated using immunofluorescent techniques to localize antigen in situ in organ dissections and cryostat sections of infected mosquitoes. The sites of restriction of replication (if restriction exists) of the vaccine strains would be defined by the comparative IF studies of antigen development in organs of mosquitoes.

A further obstacle to assessment of vector competence has been the lack of a suitable laboratory animal to use to detect mosquito transmission of low passage or attenuated dengue viruses. Development of an in vitro assay which permitted assay of transmission by inoculation of collected mosquito saliva into recipient mosquitoes was a major advance (Aitken, 1979; Beaty and Aitken, 1979). This technique facilitated transmission assays for viruses that did not cause observable morbidity or mortality in animals. Unfortunately, mosquitoes could not always be induced to engorge upon the artificial meal system used to capture the saliva. Refinement by Spielman and Possignol (unpublished data) of a saliva capture technique using oil-charged capillaries (Burlbut, 1966), provided a possible new in vitro technique to assay for virus transmission. Studies were begun to determine if the technique could be applied to the comparisons of dengue parent and vaccine viruses in mosquitoes.

The combination of transmission and comparative pathogenesis studies and the determination of dose-response curves (thresholds of infection) were thought to be adequate to reveal differences in vector-virus interactions between parental and vaccine viruses.

IV. Methods and Materials

A. Viruses:

Dengue 2

Stock viruses for both the parental and S-1 vaccine strains of dengue-2 virus were prepared in either LLC-MK_2 or Aedes albopictus C6/36 cells. The original infected human serum (PR-159) was the source of the parental virus. The experimental vaccine virus (Lot #4, Jan. 1976, WRAIR) was the seed for the vaccine stocks. To prepare the tissue culture stock pools, monolayers of LLC-MK_2 cells (31°C) were inoculated with the respective seed virus. On day 7 post inoculation, fluids were harvested, centrifuged, and the supernatant was aliquoted and frozen.

Dengue 1

Initially, both parent and vaccine dengue-1 stock viruses were prepared by inoculation of LLC-MK_2 cells. Viruses were harvested after 7 days, aliquoted, and frozen. Subsequently, stocks were prepared by inoculation of Aedes albopictus C6/36 cells. Viruses were harvested after 14 days (28°C), aliquoted, and frozen.

B. Mosquitoes:

Two long-colonized vector species were used in these studies: the Amphur (Thailand) strain of Ae. aegypti and the Oahu (Hawaii) strain of Ae. albopictus. Emerged adults were allowed to feed on sugar cubes and had access to water wicks. When this procedure was followed it was not necessary to starve mosquitoes prior to their engorgement on infectious bloodmeals; generally greater than 80% of the mosquitoes exposed became fully engorged. The mosquitoes were maintained at 28°C and approximately 80% RH.

C. Conjugate:

The anti-dengue-1 or -2 conjugate was prepared by hyperimmunization of mice (Brandt et al., 1967). Immunoglobulins were precipitated from the ascitic fluids with $(NH_4)_2SO_4$ and conjugated with fluorescein isothiocyanate (Spendlove, 1966; Hebert et al., 1972). Conjugated antibodies were purified by Sephadex G-50 column chromatography. The conjugate titered 1:32 and was used at 1:16.

D. Virus Assay:

Titrations - For the dengue-2 titrations, serial 10-fold dilutions of infectious bloodmeals were inoculated into 8 well Lab-Tek slides seeded with BHK-21 cells. Four days post-inoculation the slides were examined for viral antigen by IF. Alternatively, serial 10-fold dilutions of the dengue-2 preparations were inoculated intrathoracically into uninfected Ae. aegypti mosquitoes (10 per dilution, 0.0006 ml per mosquito). Inoculated mosquitoes were held 7-10 days (28°C) at which time heads were severed, squashed on slides, and examined for viral antigen by IF (Kuberski and Rosen 1977).

For dengue-1 parent and vaccine all titrations were done using Lab-Tek slides seeded with Aedes albopictus C6/36 cells. Serial 10-fold dilutions of the preparations were inoculated into the plates. After 7 days incubation (28°C), slides were examined by IF for the presence of viral antigen.

Antigen detection - IF was used to localize viral antigen in situ in organ dissections and cryostat sections of mosquitoes (Beaty and Thompson, 1976, 1978) and in head and abdominal squash preparations (Kuberski and Rosen, 1977).

E. Oral Transmission

After an extrinsic incubation period of 14-21 days mosquitoes were allowed to engorge individually on a serum-sucrose-red-dye drop (approximately 0.05ml). After engorgement of mosquitoes, the residual drop was inoculated into 7-10 uninfected mosquitoes. After incubation at 28°C for 10 days the recipient mosquitoes were head-squashed and examined by IF. Detection of viral antigen in the head tissues of the recipient mosquitoes was interpreted as a transmission of dengue virus.

F. Oral Infection of Mosquitoes

Parental and vaccine viruses were each inoculated into flasks of LLC-MK_2 or Ae. albopictus C6/36 cells (28°C). After incubation periods of 7-10 days for the dengue-2 viruses and 14 days for dengue-1 viruses, cells were detached from the flasks with rubber policemen, and the cell fluid suspensions were centrifuged at 500xg for 10 minutes. The cell pellet was resuspended in 1 ml of the remaining fluid, and combined with 1 ml of washed human red blood cells and 0.5 ml of 10% sucrose in heat-inactivated calf serum. Drops of this artificial bloodmeal were placed on the screening of cages holding mosquitoes. Engorged mosquitoes were removed and maintained at 28°C and 65-75% RH for 14-24 days.

V. Results

A. Dengue-2 studies: (See Miller et al 1982).

1. Growth curves:

Mosquitoes were permitted to engorge blood meals containing approximately 7.2 $\log_{10}$ mosquito infectious dose $(MID)_{50}$ per ml of either the parent or the vaccine dengue-2 virus. On days 3,5,7,9,11, and 14 post-feeding, 5 females

which had engorged on the parent and 5 females which had engorged on the vaccine virus were frozen at -70°C and subsequently titered for virus content by mosquito inoculation.

The virus growth curves for orally infected Ae. aegypti mosquitoes are presented in Figure 1. Titration of 5 mosquitoes immediately after exposure to bloodmeals containing 7.2 $\log_{10}MID_{50}$/ml of virus resulted in a geometric mean titer of 4.7 $\log_{10}MID_{50}$/ml for the parent PR-159 virus and 5.0 $\log_{10}MID_{50}$/ml for the attenuated S-1 virus. Titers fell on day 3 post feeding and increased to day 7. Thirty mosquitoes were fed on the respective virus strains and titrated on days 3 to 14 post-feeding. Of those that engorged the meal containing the parent virus, 27 (90%) became infected; 18 (60%) engorging the vaccine virus became infected. In general, the parent strain replicated to higher titers and more quickly in the mosquitoes than the vaccine strain (Figure 1).

2. Comparative susceptibility of Aedes aegypti and Aedes albopictus

To determine the comparative susceptibility of the two main vector species of dengue-2, Aedes aegypti and Aedes albopictus mosquitoes were permitted to engorge upon serial 10-fold dilutions of the parent and vaccine viruses (Table 1). After 14 days extrinsic incubation, mosquitoes were examined by IF for the presence of viral antigen.

Aedes albopictus mosquitoes seemed to be more susceptible than Aedes aegypti to oral infection by both the parent and vaccine viruses. Parent virus antigen was detected in 97% (68/70) and 66% (46/68), respectively, of the Aedes albopictus and Aedes aegypti that engorged the parent virus. Vaccine virus antigen was detected in 65% (40/65) and 20% (18/88) respectively of the Aedes albopictus and Aedes aegypti that engorged the vaccine virus (Table 1).

3. Threshold of infection studies

We attempted to determine the comparative threshold of oral infection for the 2 viruses in Ae. aegypti. For these experiments mosquitoes were allowed to engorge on 10-fold dilutions of the original stock virus preparations. After 14-21 days extrinsic incubation at 28°C, mosquito heads and abdomens were severed, squashed, and examined by IF for the presence of viral antigen. Detection of viral antigen in abdominal tissues indicated that the mosquito midgut had become infected. Detection of viral antigen in head tissues indicated that the midgut had become infected and that virus had subsequently disseminated from the midgut to infect secondary target organs. To determine the precise anatomic location of virus, organ systems were dissected from selected mosquitoes and examined by IF for the presence of viral antigen.

The results of the comparative oral infection experiments are presented in Tables 2 and 3. Dengue-2 viruses grew to higher titers in C6/36 than in LLC-MK_2 cells. When Ae. aegypti mosquitoes ingested the parent virus grown in C6/36 cells at titers ranging from 4.2 to 8.2 $\log_{10}MID_{50}$/ml, 74% (145/194) became infected; 97% (141/145) of the infected mosquitoes developed a disseminated infection (Table 2). In contrast when mosquitoes fed on the same titer of vaccine virus grown in C6/36 cells, 21% (39/183) became infected; 59% (23/39) of the infected mosquitoes developed a disseminated infection. The overall rate of

virus dissemination to mosquito head tissues was 72% (141/194) for the parent virus and 12% (23/183) for the vaccine virus. When the infectious titer of virus grown in C6/36 cells was 5.2-6.2 $\log_{10}MID_{50}$/ml, 67% (35/52) of the mosquitoes exposed to the parent virus became infected in contrast to 6% (4/60) exposed to the vaccine virus. The mosquito 50% oral infectious dose (OID_{50}) for the parent virus was computed to be 5.4 $\log_{10}MID_{50}$/ml and 7.2 $\log_{10}MID_{50}$/ml for the attenuated vaccine virus.

Overall infection rates were obtained by combining the results obtained using virus stocks prepared in C6/36 cells with those obtained using virus stocks grown in LLC-MK_2 cells. The total infection rate for mosquitoes ingesting bloodmeals containing 3.7 to 8.2 $\log_{10}$ MID_{50} per ml of the parent virus was 56% (220/396); in contrast, only 16% (66/397) of those ingesting the same amount of the vaccine virus became infected.

4. Oral Transmission of dengue-2 viruses

Since the attenuated vaccine virus had been demonstrated capable of infection of Ae. aegypti mosquitoes, it was necessary to determine if vaccine virus could be transmitted by mosquito bite. Mosquitoes were allowed to engorge on infectious bloodmeals containing approximately 7.2 $\log_{10}MID_{50}$/ml of either parent or attenuated dengue-2 virus (Table 4). All (22/22) of the Ae. aegypti feeding on the parent virus became infected and developed disseminated infections by 21 days extrinsic incubation. Fifty-five per cent (16/29) of the mosquitoes engorging on the attenuated virus bloodmeal became infected, but only 28% (8/29) developed a disseminated infection. Fourteen per cent (3/22) of the mosquitoes infected with the parent strain transmitted virus to a serum-sucrose drop. None of the mosquitoes infected with the vaccine strain transmitted.

5. Pathogenesis studies:

A number of mosquitoes infected with the vaccine virus were dissected in order to ascertain which tissues/organs were involved in virus replication. In many cases, viral antigen was found in large amounts in the mesenteral tissues only. The fore and hindguts as well as ovaries, ventral nerve chord, salivary glands and fat body were free of demonstrable S-1 viral antigen. It would appear that although virus was replicating in the midgut, it was unable to mature and escape into the haemocoel or unable to attach and replicate in secondary organ systems. The molecular basis for this attenuation is not known.

6. S-1 Vaccine reversion studies

Studies were conducted to determine if the S-1 vaccine virus would revert to virulence during mosquito passage. Two biological markers, plaque size and temperature sensitivity were used originally to characterize the attenuated virus. The S-1 clone produced small plaques and did not grow at temperatures of 39°C or higher. We used these markers to address the possibility that the S-1 virus might revert to virulence (large plaque size and growth at 39°C) after passage in mosquitoes. The dengue-2 viruses were characterized in the infectious bloodmeal and after growth in orally infected mosquitoes (Table 5). The S-1 cloned virus remained temperature sensitive when grown in C6/36 cells or LLC-MK_2 cells and after passage in mosquitoes. Plaque sizes were heterogeneous,

although no large plaques were seen. Surprisingly the parent virus apparently became attenuated (temperature sensitive) after passage in the C6/36 cells, and the attenuation seemed to be accentuated by passage in the mosquito vector.

B. Dengue 1 Studies

1. Infection of Aedes albopictus

To determine the relative capability of the dengue-1 parent and vaccine viruses to replicate in Aedes albopictus, mosquitoes were intrathoracically inoculated. Each day post infection, 5 mosquitoes were removed and frozen for subsequent titration in C6/36 cells. Preliminary results are shown in Table 6. Both viruses replicated well after intrathoracic infection. Endpoints have not yet been reached for most mosquitoes. Nonetheless the results suggest that the parent virus may be more efficient in replication in the vector than the vaccine virus. Most mosquitoes infected with ≤ 1.0 $\log_{10}$ $TCID_{50}$ of the parent virus titered ≥ 5.0 $\log_{10}$ $TCID_{50}$ after 4 days extrinsic incubation. In contrast, most mosquitoes infected with 300 times as much vaccine virus titered between 4.0 and 5.0 $\log_{10}$ $TCID_{50}$.

2. Oral infection of Aedes albopictus

Studies were conducted to determine the comparative ability of parent and vaccine viruses to orally infect Aedes albopictus. Mosquitoes were permitted to engorge meals containing freshly prepared virus stocks as described previously for dengue-2 feeds. On selected days post-infection, mosquitoes were stored for subsequent titration. Unfortunately after a one-week incubation period at 28°C, the parent virus meal titered approximately 3.0 $\log_{10}$ $TCID_{50}$ per ml. Apparently this titer was below the threshold of infection for the mosquitoes; none that engorged the meal prepared using this stock became infected. The problem with low parent virus meal titers has been overcome by preparation of the stocks in Aedes albopictus cell cultures. After 2 weeks incubation at 28°C, titers of 9.0 $\log_{10}$ $TCID_{50}$/ml are achieved.

In this first study, the dengue vaccine virus meal preparation titered 5.8 $\log_{10}$ $TCID_{50}$/ml. This titer did result in mosquito infection. Preliminary results are shown in Table 7. Progeny virus was not detected in mosquitoes in more than trace amounts until 7 days post-engorgement. Of the 24 mosquitoes so far examined after 7 to 14 days extrinsic incubation, only 10 (42%) became infected.

3. Threshold of infection studies

To determine the comparative threshold of oral infection for the 2 viruses, Aedes aegypti mosquitoes were permitted to engorge upon meals containing serial 10-fold dilutions of the stock virus preparations. After 14 days extrinsic incubation at 28°C, mosquitoes were processed by IF for the presence of viral antigen. The parent meal titered 8.0 $\log_{10}$ $TCID_{50}$ per ml, and the vaccine meal titered 7.75 logs. The Aedes aegypti mosquitoes only became infected when they engorged the meal prepared from the undiluted virus. The disseminated infection rates were 30% (14/46) for mosquitoes engorging the parent virus and 39% (12/31) for those engorging the vaccine virus. The rates do not differ statistically.

The low infection rates are surprising. After ingestion of considerably less dengue-1 vaccine virus (Table 7), 42% (10/24) of Aedes albopictus mosquitoes contained detectable virus after 7 days extrinsic incubation. This would seem to indicate that the Aedes albopictus mosqitoes are more susceptible to both dengue-1 and -2 (Table 1) viruses.

C. Development of Improved Oral Transmission Assay

Studies were begun to determine if the oil in vitro transmission assay could be successfully applied to detect dengue virus transmission.

Mosquitoes were inoculated with either dengue-2 parent or vaccine virus, yellow fever virus, or La Crosse virus. After 1 week incubation, wings and legs were removed from the mosquitoes and the probosci were inserted into capillary pipettes charged with 3.5 ul of Cargille immersion oil. After 30-60 minutes exposure, mosquitoes were removed and examined by IF for the presence of viral antigen. Charged capillaries containing the mosquito saliva were placed in Eppendorf centrifuge tubes containing 0.1ml of 20% FCS-PBS diluent. The tubes were centrifuged twice for 1 minute in order to force the contents of the capillary into the diluent. Centrifuge tubes were then frozen. To assay for virus transmission, the contents of the tubes were subsequently inoculated into recipient mosquitoes. After 14 days, recipient mosquitoes were headsquashed and processed by IF. Detection of antigen indicated virus transmission.

To compare the in vitro technique to in vivo transmission, sibling mosquitoes, infected with either yellow fever or La Crosse virus, were separated into two groups. One group of each was permitted to engorge upon suckling mice; the other was assayed for transmission using the in vitro technique (Tables 8 and 9).

Transmission of both yellow fever and La Crosse virus was demonstrated. In these pilot studies (Tables 8 and 9), some difficulties were encountered with mouse and recipient mosquito survival. Nonetheless, the results were encouraging. Interestingly, after 1 week extrinsic incubation, 4 mosquitoes without detectable viral antigen in the headsquash preparation transmitted virus (Table 8). Thus it seems that the assay can detect transmission before sufficient viral antigen to detect by IF accumulates in the head tissues. Since the mosquitoes were inoculated parenterally, all presumably were infected. In those instances where the in vivo and in vitro techniques could be compared, yellow fever transmission rates were similar.

Similar results were obtained with La Crosse virus transmission attempts (Table 9). Only 1 mouse survived after being fed upon by an infected Aedes triseriatus mosquito. Serum has been collected from the mouse but not yet tested for the presence of antibodies to La Crosse virus. Three of the remaining 4 (75%) mice fed upon survived Table 9. After a similar 2 weeks incubation, 9 of 10 (90%) of the mosquitoes assayed using the in vitro transmission technique were demonstrated to have transmitted.

On the basis of these results, a pilot study was conducted to compare the

transmission rates of the parent and vaccine dengue-2 viruses using the in vitro technique. Results are shown in Table 10. Transmission of both viruses was detected. Interestingly, the mosquitoes inoculated with the parent virus received approximately 0.8 $\log_{10}$ TCID $_{50}$; whereas the mosquitoes infected with the vaccine virus received approximately 2.6 logs. Nonetheless, after 1 week extrinsic incubation, transmission rates by parent and vaccine infected mosquitoes were similar.

Further studies are planned to clearly delineate the extrinsic incubation periods as well as effective duration and rates of transmission of the parent and vaccine viruses. The use of the improved oil in vitro assay permitted testing of mosquitoes in a fraction of the time necessary using the old in vitro assay. If the technique can be demonstrated to be as sensitive and specific for salivary gland transmission as an in vivo assay, it will greatly facilitate the proposed studies.

VI. Discussion

The S-1 vaccine strain seemed to be markedly less efficient than the parent PR-159 strain in interactions with potential vector species. The S-1 vaccine was considerably less efficient in oral infection of vectors (Tables 1,2, and 3); it was considerably less efficient in developing disseminated infection (see progress report, 1980, Tables 2 and 3); when disseminated infection did occur, it was later than that for the PR-159 strain; and finally the vaccine strain was less efficiently transmitted (Table 4).

Thus we conclude that dengue-2, S-1 vaccine virus, which is attenuated for man and animals is also modified in its ability to infect orally and to be transmitted by Ae. aegypti mosquitoes. Oral infection only occurred in a substantial number of mosquitoes when the infectious titer of the meal was relatively high (Tables 1 and 2). The vaccine strain was approximately 100 times less efficient than the parent strain in orally infecting Ae. aegypti. Although viremia does occur in humans inoculated with the vaccine, it does so at levels so low that the virus must be first amplified in cell culture before it can be recovered. Nonetheless, a few Aedes aegypti mosquitoes did become infected while feeding on viremic vaccinees. However, none of the infected mosquitoes contained detectable virus antigen in head tissues, nor was virus transmitted by an infected mosquito (Bancroft et al, 1982). Likewise, in the studies reported herein, none of the Ae. aegypti mosquitoes orally infected with the vaccine virus subsequently transmitted. It seems reasonable to speculate that the virus infection in those mosquitoes that fed on the vaccines was restricted to the midgut.

The Aedes albopictus mosquitoes seemed to be more vector competent (oral infection) than the Aedes aegypti mosquitoes (Table 1). However, it must be noted that these observations were made using highly laboratory adapted strains of mosquitoes. Data derived using these two laboratory strains should not necessarily be extrapolated to species differences in vector competence in nature. Since genetic variability in vector competence of Ae. aegypti and Ae. albopictus populations has been demonstrated (Gubler and Rosen 1976; Gubler et. al., 1979), similar studies should be conducted with selected, epidemiologically significant geographic strains of these mosquitoes.

The attenuated virus remained temperature sensitive after replication in mosquitoes. This is not surprising since the mosquitoes were maintained at temperatures well below 39°C. The plaque morphology was not uniformly small, although large plaques characteristic of the parent virus were not detected. Temperature sensitivity and plaque size/morphology are biological markers which may or may not be related or correlated with the parameters of vector competency. In this particular case, the inability of the S-1 vaccine virus to infect efficiently and to be transmitted by Ae. aegypti mosquitoes is certainly a relevant albeit complex biological marker.

Preliminary indications based on the limited replication and oral infection studies suggest that the dengue-1 vaccine is also modified in its ability to interact with vector mosquitoes. Perhaps there is a common basis for attenuation of flaviviruses.

VII. Conclusions

1. The dengue-2, S-1 vaccine would seem to be sufficiently modified in its ability to infect and to be transmitted by vector mosquitoes to preclude secondary infections as a result of mosquitoes becoming infected by recent vaccinees. Importantly, even in the unlikely event that mosquito infection and transmission did occur, the virus probably would not revert to virulence during mosquito passage.

The S-1 vaccine virus was less efficient than the parent virus in the following vector-virus interactions:

a) replication in intrathoracically or orally infected mosquitoes.
b) oral infection of vector mosquitoes.
C) oral transmission by vector mosquitoes.

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Table 1

Infection rates for Aedes aegypti and Aedes albopictus orally infected with graded doses of dengue-2 parent and vaccine viruses

Titer of feeding suspension $log_{10}TCID_{50}$/ml	Parent (PR-159)		Vaccine (S-1)	
	Aedes aegypti	Aedes albopictus	Aedes aegypti	Aedes albopictus
8.2 - 7.2	3 (100)[1]	15 (100)	8 (100)	20 (95)
7.2 - 6.2	21 (95)	25 (100)	25 (28)	17 (65)
6.2 - 5.2	20 (65)	18 (95)	29 (7)	14 (57)
5.2 - 4.2	24 (25)	11 (91)	26 (4)	11 (18)
TOTAL				
8.2 - 4.2	68 (66)	70 (97)	88 (20)	62 (65)

[1] Number tested (percent positive).

Table 2

Infection rates of Aedes aegypti mosquitoes orally infected with graded doses of dengue-2 parent and attenuated viruses grown in C6/36 cells.

Titer of bloodmeal[1]	Parent virus (PR-159)		Vaccine virus (S-1)	
	Infected[2] (%)	Disseminated[3] (%)	Infected (%)	Disseminated (%)
8.2-7.2	55/56 (98)	55/56 (98)	8/8 (100)	8/8 (100)
7.2-6.2	41/42 (98)	41/42 (98)	25/55 (45)	11/55 (20)
6.2-5.2	35/52 (67)	35/52 (67)	4/60 (7)	3/60 (5)
5.2-4.2	14/44 (32)	10/44 (23)	2/60 (3)	1/60 (2)
Total	145/194 (75)	141/194 (73)	39/183 (21)	23/133 (13)

[1] Log_{10} mosquito infective dose 50 per ml.
[2] Number mosquitoes positive for dengue-2 viral antigen in midgut/number tested.
[3] Number mosquitoes positive for dengue-2 viral antigen in head tissues/number tested.

Table 3

Infection rates of Aedes aegypti mosquitoes orally infected with graded doses of dengue-2 parent and attenuated viruses grown in LLC-MK_2 cells

Titer of bloodmeal[1]	Parent virus (PR-159)		Vaccine virus (S-1)	
	Infected[2] (%)	Disseminated[3] (%)	Infected (%)	Disseminated (%)
7.2-5.7	36/49 (73)	32/49 (65)	20/60 (33)	8/60 (13)
5.7-4.7	30/50 (60)	26/50 (52)	6/68 (9)	3/68 (4)
4.7-3.7	8/59 (14)	5/59 (8)	1/42 (2)	1/42 (2)
3.7-2.7	1/44 (2)	1/44 (2)	0/44 (0)	0/44 (0)
[illegible]	75/202 (37)	64/202 (32)	27/214 (13)	12/214 (6)

[1] Log_{10} MID_{50} per ml.
[2] Number mosquitoes positive for dengue-2 viral antigen in midgut/number tested.
[3] Number mosquitoes positive for dengue-2 viral antigen in head tissues/number tested.

Table 4

Infection and transmission rates for Aedes aegypti mosquitoes orally infected with dengue-2 parent and attenuated viruses.

	Dengue-2 viruses[1]	
	Parent virus (PR-159)(4)	Vaccine virus (S-1)(4)
No. mosquitoes exposed	22	29
No. infected	22 (100)	16 (55)
No. disseminated[2]	22 (100)	8 (28)
No. transmitting	3 (14)	0 (0)

[1] Dengue-2 viruses were grown in LLC-MK_2 cells at 31°C; post-feeding titer was 7.2 $\log_{10}$ MID_{50}/ml for PR-159 and S-1 viruses.

[2] Dengue-2 viral antigen detected in mosquito head tissues.

Table 5

Plaquing of dengue-2 parent (PR-159) and attenuated (S-1) viruses at permissive and restrictive temperatures before and after oral passage in Aedes aegypti mosquitoes.

Sample	PFU[1]/0.2 ml. 35°C	38.5°C	39.3°C
1. S-1 grown in $C_6/36$ cells at 27°C	5.1×10^5	4.4×10^3	<10
2. S-1 from Ae. aegypti orally infected with #1	2.2×10^4	2.8×10^2	<10
3. S-1 grown in LLC-MK_2 cells at 31°	5.1×10^3	1.7×10^2	<10
4. S-1 from Ae. aegypti orally infected with #3	4.9×10^3	3.0×10^1	<10
S-1 control	3.4×10^5	2.3×10^3	<10
5. Parent grown in $C_6/36$ cells at 27°	4.5×10^6	1.3×10^5	8.4×10^3
6. Parent from Ae. aegypti orally infected with #5	1.0×10^3	<10	<10
Parent Control	1.7×10^6	6.7×10^5	3.2×10^5

[1] Samples #1-6 all contained dengue-2 virus that resulted in heterogeneous plaque sizes ranging from 0.5 mm. to 1.5 mm. except sample #5 which contained plaques that were <2.0 mm in size. None of the samples (# 1-6) contained the large plaques seen in the PR-159 control.

Table 6

Replication of dengue-1 viruses after intrathoracic inoculation[1]

into Aedes albopictus Mosquitoes

Virus	Mosquito	Titer of virus ($TCID_{50}$) Days post infection										
		0	1	2	3	4	5	6	7	8	...	16
dengue-1	1	<1.0	<1.0	<1.0	4.25	>5.0	>5.0	>5.0	>5.0	>5.0		>5.0
Parent[1]	2	<1.0	<1.0	<1.0	3.75	>5.0	>5.0	>5.0	>5.0	>5.0		>5.0
	3	<1.0	<1.0	3.0	3.75	>4.0	>5.0	>5.0	>5.0	>5.0		
	4	<1.0	<1.0	2.25	4.0	>5.0	>5.0	>5.0	>5.0	>5.0		
dengue-1	1	<1.0	<1.0	<1.0	3.75	>4.0	>4.0	>5.0	>5.0	>5.0		>4.0
Vaccine[2]	2	<1.0	<1.0	<1.0	4.0	>5.0	>4.0	>5.0	>4.0	>5.0		>5.0
	3	<1.0		<1.0	3.5	>4.0	>4.0	>5.0	>5.0	>5.0		>5.0
	4	<1.0		<1.0	2.25	>4.0	>4.0	>4.0	>5.0	>4.0		>5.0

1 Dengue-1 parent inoculum titered <1.0 $\log_{10}$ $TCID_{50}$ per ml.
2 Dengue-1 vaccine inoculum titered 2.6 $\log_{10}$ $TCID_{50}$ per ml.

Table 7

Replication of dengue-1 vaccine virus (TP56)

after oral infection of Aedes albopictus mosquitoes

	Day post infection												
Mosquito	0	1	2	3	4	5	6	7	8	9	10	11	14
1	2.25	-	<2.0	<1.0	-[1]	-	< 1.0	2.0[2]	2.75	3.25		5.0	-
2	2.75	-	-	<1.0	-	-	-	-	2.0	3.75		-	3.5
3	2.5	-	-	-	-	-	-	-	-	3.25		-	4.0
4	3.0	-	-	<1.0	<1.0	-	-	-	-	-		-	-
5													4.0
6													-
7													3.0
8													-

[1] no virus detected in titration

[2] Log_{10} $TCID_{50}$ per mosquito

Table 8

Comparison of an in vitro technique and engorgement upon suckling mice for an assay of yellow fever virus transmission by mosquitoes.

	In Vitro				Suckling Mice			
	Donor incubation period				Donor incubation period			
	1 week		2 weeks		1 week		2 weeks	
No.	Donor	Recip.	Donor	Recip.	Donor	mouse death	Donor	mouse death
1	+[2]	+	+	+	+	+	+	NF[3]
2	-	-	+	NS[2]	+	-	+	+
3	+	+	+	NS	+	+	+	NF
4	+	NS	+	NS	-	-	+	+
5	+	+	+	NS	+	-	+	NF
6	+	+	+	NS	+	+	+	NF
7	+	NS	+	NS	+	-	+	NF
8	-	+	+	+	+	-	+	NF
9	+	+	-	-	+	+		
10	+	+	-	-	+	+		

[1]Results of IF examinations of headsquash preparations.

[2]None of the recipients survived the two weeks incubation period.

[3]Mosquito did not feed on mouse.

Table 9

Comparison of an in vitro technqiue and engorgement upon suckling mice for an assay of La Crosse virus transmission by mosquitoes.

	In Vitro				Suckling Mice			
	Donor incubation period				Donor incubation period			
	1 week		2 weeks		1 week		2 weeks	
No.	Donor	Recip.	Donor	Recip.	Donor	mouse death	Donor	mouse death
1	+[1]	+	+	+	+	D[3]	+	NF[4]
2	+	+	+	+	+	D	+	+
3	+	+	+	+	+	D	+	NF
4	+	NS[2]	+	+	-	D	+	NF
5	+	NS	+	+	+	D	-	NF
6	+	+	+	+	+	D	+	NF
7	+	+	+	+	+	D	+	NF
8	+	+	+	-	+	D	+	-
9	+	NS	+	+	+	D	+	+
10	+	NS	+	+	+	D	+	+

[1]Results of IF examination of headsquash preparations.

[2]None of the recipients survived the one week incubation period.

[3]Non-virus associated mouse death.

[4]Mosquito did not feed on mouse.

Table 10

In vitro transmission of dengue-2 parent and vaccine viruses using the oil capillary technique.

	Parent				Vaccine			
	Donor incubation period				Donor incubation period			
	1 week		2 weeks		1 week		2 weeks	
No.	Donor	Recip.	Donor	Recip.	Donor	Recip.	Donor	Recip.
1	+[1]	+	+	+	+	+	+	-
2	+	-	+	-	+	NS	+	+
3	+	+	+	+	+	NS	+	+
4	-	+	+	+	+	-	+	NS
5	-	NS[2]	+	+	+	-	+	+
6	+	NS	+	+	+	NS	+	NS
7	-	NS	+	+	+	+	+	NS
8	+	NS	+	+	+	+	+	+
9	+	+	+	+	+	NS	+	+
10	+	+	-	+	+	NS	+	+

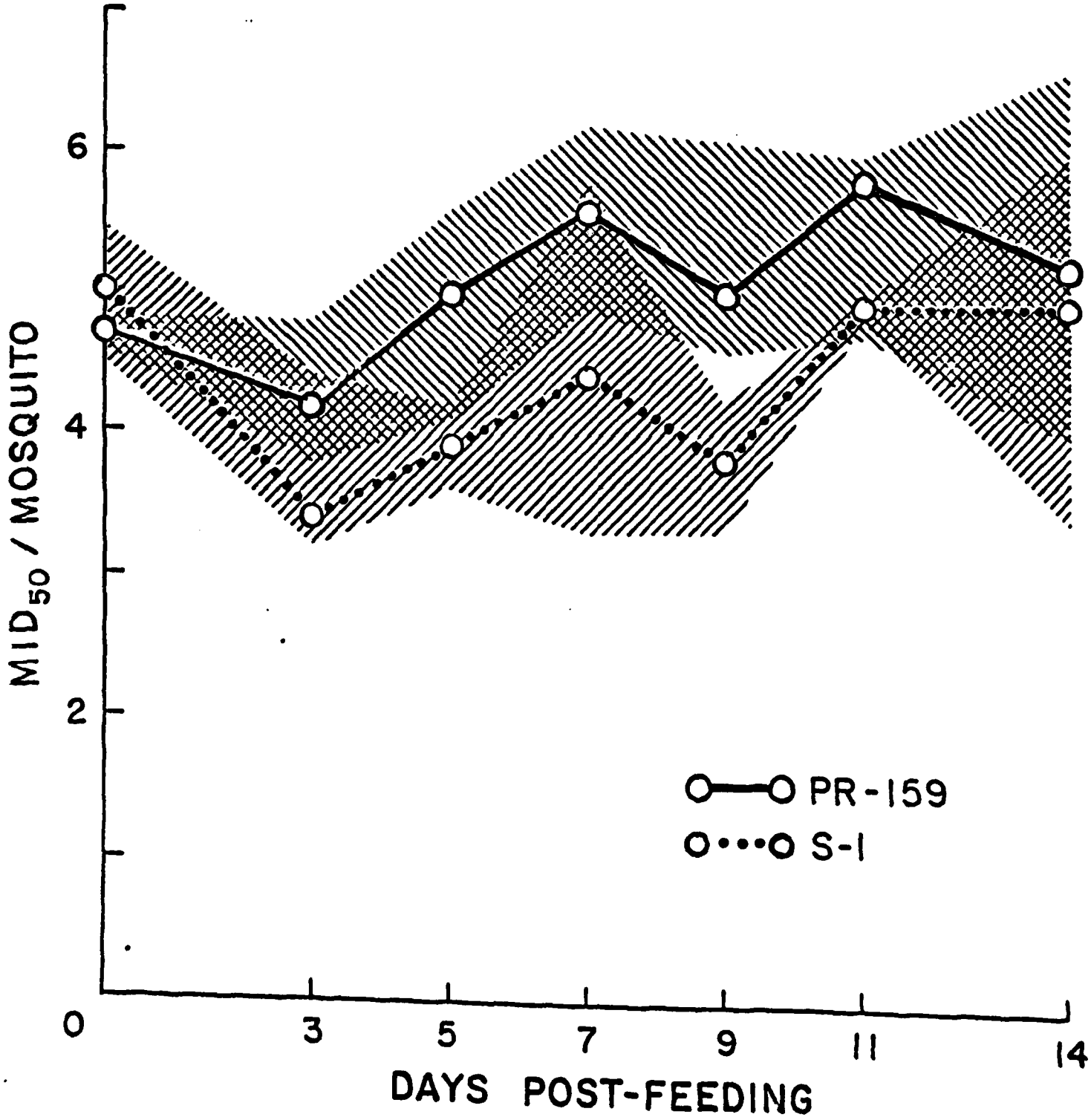
[1]Results of IF examinations of headsquash preparations.

[2]None of the recipients survived the two week incubation period.

Figure 1

Replication of dengue-2 parent (PR-159) and vaccine (S-1) viruses in *Aedes aegypti* mosquitoes.[1,2]

1 Crosshatched area indicates range of titers.
2 Dengue-2 viruses were grown in LLC-MK_2 cells at 31^0C; post-feeding titer of the blood meals were 7.2 MID_{50} per ml for both the parent and vaccine viruses.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